Mutation of Lys-75 affects calmodulin conformation

Marina V. Medvedeva[a], Oxsana V. Polyakova[a], D. Martin Watterson[b], Nikolai B. Gusev[a,*]

[a] *Department of Biochemistry, School of Biology, Moscow State University, Moscow 119899, Russia*
[b] *Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, Chicago, IL, USA*

Received 22 March 1999

Abstract **Some properties of synthetic calmodulin and its five mutants with replacement of Lys-75 were analyzed by means of electrophoresis, limited proteolysis and MALDI mass-spectrometry. A double mutant of calmodulin containing insert KGK between residues 80 and 81 and replacement of Lys-75 by Pro has a highly flexible central helix which is susceptible to trypsinolysis in the presence of Ca^{2+}. Two mutants, K75P and K75E, having a distorted central helix demonstrate high resistance to trypsinolysis in the absence of Ca^{2+}. Arg-90 and Arg-106 being the primary site of trypsinolysis of synthetic calmodulin are partially-protected in K75P and K75E mutants. The central helix of K75A and K75V mutants is stabilized by hydrophobic interactions between residues located in positions 71, 72 and 75. In the presence of Ca^{2+}, the central helix of K75V is resistant to trypsinolysis. Mutations K75A and K75V decrease the rate of trypsinolysis of the central helix with a simultaneous increase of the rate of trypsinolysis in the C-terminal domain of calmodulin. It is concluded that the point mutation in the central helix has a long distance effect on the structure of calmodulin.**

Key words: Calmodulin; Mutant; Limited proteolysis; Mass-spectrometry

1. Introduction

Calmodulin (CaM) is a ubiquitous Ca-binding protein, regulating the activity of many intracellular proteins in eukaryotic cells [1]. Because this single protein is involved in the regulation of a diverse array of enzymes, structural proteins and membrane transporters detailed knowledge about how the CaM structure is related to its calcium-binding activity is required for an understanding of how eukaryotic cells integrate their differential responses to intracellular calcium signals.

In the crystal, CaM has a dumbbell-like form consisting of two globular domains each containing two Ca-binding sites and a long α-helix connecting these globular domains [2]. In solution, part of the central helix (residues 74–82) adopts a non-helical conformation and therefore, this helix behaves as a flexible tether connecting two globular Ca-binding domains of CaM [3–5]. It is generally accepted that there is an ordered binding of calcium and that there is cooperativity between each pair of sites in two globular domains [1,6]. The controversy is whether CaM is composed of two coupled globular domains connected by a functional central region or two independent pairs of cooperative sites connected by a passive tether. According to the later model, the central helix of CaM serves a more passive role and is not directly involved in the coupling of function between the two globular domains. However, studies have supported the model of two coupled domains, including changes in susceptibility to proteolysis [7], functional effects of site-directed mutagenesis [8], site-specific probes [9], scanning calorimetry [10] and the involvement of the central helix residues in interaction with peptides [11]. Therefore, a variety of experimental data are consistent with a model of two coupled domains and an active role for the flexible central helix.

In order to further test this model and directly address the potential effects of the central helix bend region of CaM, we analyzed some properties of the synthetic protein hybrid of mammalian and plant calmodulin [12], SynCam, and its five mutants with replacement of Lys-75 [13]. Combining the use of mutant CaM, limited proteolysis and MALDI mass-spectrometry (MALDI-MS), we demonstrated that a point mutation in position 75 induces long distance effects on the structure of CaM. These changes in CaM structure may affect its interaction with different target proteins.

2. Materials and methods

Expression of SynCaM and its mutants and their purification was performed according to earlier described methods [12,14]. Wild-type CaM was isolated from bovine brain according to the method of Gopalakrishna and Anderson [15]. Final preparations of CaM were freeze-dried and kept at −20°C until used. The purity of protein samples was checked by 15% polyacrylamide gel electrophoresis, which was run both in the absence and in the presence of 6 M urea [16], or by SDS-gel electrophoresis [17]. The concentration of the wild-type CaM was determined spectrophotometrically using $E_{280}^{0.1\%} = 0.2$ cm^{-1} [18]. The concentration of SynCaM and its mutants was determined by a dye-binding assay [19] using bovine brain CaM as a standard.

Trypsinolysis of CaM (1.5–2 mg/ml) was performed in 20 mM NH_4HCO_3 in the presence of 2 mM EGTA or 1 mM $CaCl_2$ at 25°C. In the absence of Ca^{2+} (2 mM EGTA), the weight ratio CaM/trypsin was equal to 500/1 and incubation lasted for 12 min, whereas in the presence of Ca^{2+} (1 mM $CaCl_2$), the weight ratio CaM/trypsin was equal to 25/1 and incubation lasted for 30 min. Trypsinolysis was started by the addition of TPCK-treated trypsin (Sigma) and stopped by addition of either phenylmethylsulfonyl fluoride (up to the final concentration of 1 mM) or by addition of trifluoroacetic acid (up to the final concentration of 2%). The mixture of tryptic peptides of CaM was subjected to native 15% polyacrylamide gel electrophoresis [16] or to MALDI-MS.

MS was performed on a Vision-2000 spectrometer (BioAnalysis). The matrix was either dihydrobenzoic acid (DHB) or ferulic acid. The samples were loaded on the target by the dried droplet method. The analysis was performed in a positive mode and around 50 scans were averaged. Spectra obtained were calibrated externally using $[M+H^+]$ ion from two protein standards, human insulin chain B: *m/z* 5807.6 and horse cytochrome *c*: *m/z* 12 384.

*Corresponding author. Fax: (7) (095) 939 39 55.
E-mail: gusev@gusev.bio.msu.su

Abbreviations: CaM, calmodulin; EGTA, ethyleneglycoltetraacetate; MALDI-MS, matrix assistant laser desorption mass-spectrometry; SDS, sodium dodecyl sulfate

0014-5793/99/$20.00
PII: S0014-5793(99)00483-4

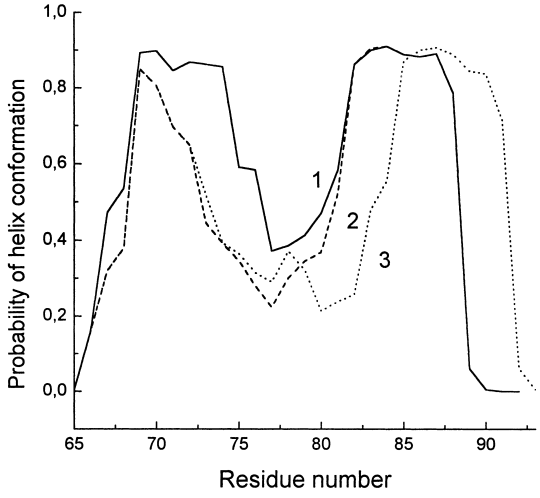

Fig. 1. Prediction of α-helix formation in the central part of SynCaM (1), VU-11 (K75P) (2) and VU-35, a double mutant of SynCaM (K75P plus KGK insert between residues 80 and 81) (3).

3. Results

In this investigation, we analyzed some properties of six CaM species differing in the length and composition of the central helix (Table 1). The central helix of SynCaM differs from the bovine brain wild-type CaM by conservative replacement of Thr-70 and Met-71 by Asn and Leu and by replacement of Ile-85 and Arg-86 by Leu and Lys, respectively [12,13] (Table 1). Other mutants analyzed contained different residues in position 75. In two cases (VU-11 and VU-35), Lys-75 was replaced by Pro. VU-57B contained Glu instead of Lys in position 75, whereas in VU-57A and VU-57D, Lys-75 was replaced by Ala or Val (Table 1).

When the peptide with the primary structure corresponding to residues Gly-61–Phe-92 of SynCaM was analyzed by the method of Garnier et al. [20], two regions with a high probability of α-helix formation were detected. Residues 69–75 and 82–88 have a high probability of α-helix formation (Fig. 1), whereas residues 77–80 form a coil. These predictions agree with the data of literature indicating the presence of a flexible hinge in the middle of the central helix [3–5]. As expected, mutation K75P (VU-11) decreases the probability of α-helix formation for residues 71–81 (Fig. 1). In the double mutant with replacement of Lys-75 by Pro and the simultaneous insert of tripeptide KGK between residues 80 and 81 (VU-35), residues 74–83 tend to form coil structure and, therefore, VU-35 seems to have a very long hinge region connecting two short α-helices (Fig. 1).

The method of Garnier et al. [20] does not consider electrostatic or hydrophobic interactions between neighboring residues. Therefore, this method cannot reveal any significant changes in the probability of α-helix formation of K75A, K75E, K75V and SynCaM (data not presented). At the same time, mutation K75E will prevent formation of the salt link between residues Lys-75 and Asp-78 presented in the wild-type CaM [2] and SynCaM. Moreover, this mutation may produce electrostatic repulsion between residues Glu-75 and Asp-78 and by this way destabilize the central helix of the K75E mutant. On the hand, replacement of polar Lys-75 by hydrophobic Ala (K75A) or Val (K75V) increases the probability of hydrophobic contacts between residue 75 and Met-71 and Met-72. This can increase the stability of the N-terminal portion of the central helix and decrease its flexibility. Thus, we may suppose that any mutations of position 75 will affect the structure of the central helix of CaM.

In the presence of Ca^{2+} under non-denaturing conditions, all CaM mutants have a similar electrophoretic mobility except of VU-35 migrating slightly slower (Fig. 2A). This is understandable since VU-35 has a slightly higher molecular

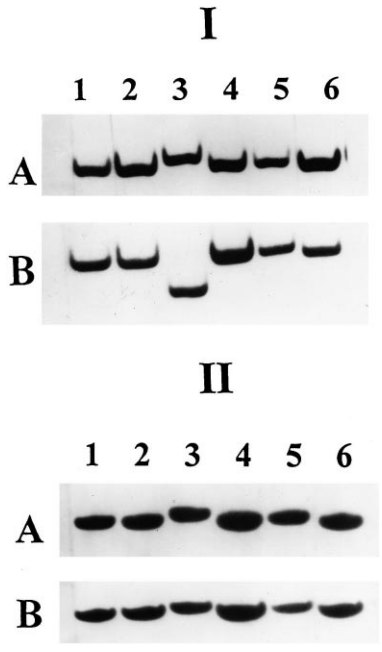

Fig. 2. Electrophoretic properties of calmodulin mutants. The mutants were subjected to electrophoresis in the presence of 1 mM $CaCl_2$ (A) or in the presence of 1 mM EGTA (B). Electrophoresis was performed in the absence of denaturing agents (I) or in the presence of 6 M urea (II). SynCaM (1), VU-11 (2), VU-35 (3), VU-57A (4), VU-57B (5), VU-57D (6).

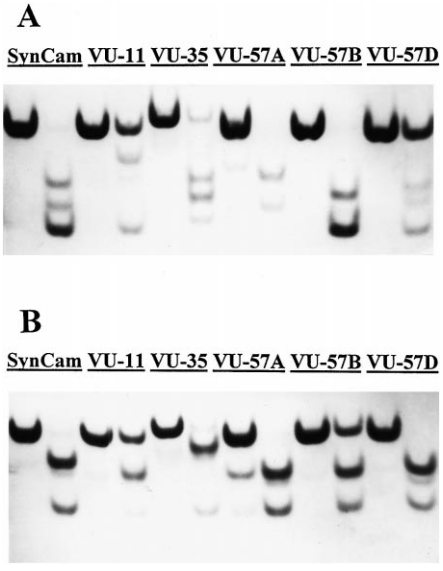

Fig. 3. Native electrophoresis of the samples obtained after limited trypsinolysis of calmodulin mutants in the presence of 1 mM $CaCl_2$ (A) or 2 mM EGTA (B). In order to compare peptide maps, all samples were run in the presence of an excess calcium. Two tracks for each protein represent calmodulin mutants before and after trypsinolysis.

weight and two additional positive charges comparing to other CaM mutants. If the native gel electrophoresis was performed in the absence of Ca^{2+}, VU-35 migrated with a much higher mobility than all other mutants of CaM (Fig. 2BB). The only explanation is that in the absence of Ca^{2+}, VU-35 has a much more compact structure than other CaM mutants. VU-35 can adopt a more compact structure due to the high flexibility of its central helix (Fig. 1). If this explanation is correct, we may expect that in the presence of urea, causing unfolding of the protein structure, all CaM mutants will have a similar electrophoretic mobility. Indeed, if electrophoresis was performed in the presence of 6 M urea, VU-35 migrated slightly slower than all other CaM mutants, both in the presence and in the absence of Ca^{2+} (Fig. 2). Thus, we may conclude that insertion of KGK between residues 80–81 of SynCaM, combined with replacement of Lys-75 by Pro, strongly affects the flexibility of the central helix and this effect is especially pronounced in the absence of Ca^{2+}.

In order to analyze the structure in more details, we subjected CaM mutants to limited trypsinolysis, both in the presence and absence of Ca^{2+}, and analyzed the peptide mixture by native gel electroptions. The used incubation with trypsin results in the complete degradation and disappearance of the band of intact SynCaM (Fig. 3). In the presence of Ca^{2+}, VU-35, VU-57A and VU-57B were cleaved by trypsin with a rate comparable to that of SynCaM (Fig. 3A). At the same time, at the end of incubation, we detected significant quantities of intact VU-11 and VU-57D (Fig. 3A). Thus, in the presence of Ca^{2+}, mutations K75P and K75V reduce the rate of trypsinolysis of CaM. It is well known that in the presence of Ca^{2+}, Arg-74, Lys-75 and Lys-77 are the main sites of tryptic attack [21,22]. Replacement of Lys-75 by Pro will prevent or decrease the rate of trypsinolysis both at position 74 and 75 and by this means, diminish the rate of VU-11 degradation. It is worthwhile to mention that VU-35, having the same replacement in position 75, is practically completely degraded in the presence of Ca^{2+} (Fig. 3A). This seems to be due to the fact that this mutant contains a highly flexible and exposed central helix with additional sites of trypsinolysis (KGK insert) which are absent in VU-11. A decreased rate of trypsinolysis of VU-57D in the presence of Ca^{2+} can be due to stabilization of the central helix of this mutant by hydrophobic contacts formed between Val-75 and Leu-71 and Met-72.

In the absence of Ca^{2+}, CaM is cleaved by trypsin with a high rate [21]. The rate of trypsinolysis of SynCaM was comparable with that of VU-35, VU-57A and VU-57D and at the end of incubation, no band of intact protein was detected on the gel (Fig. 3B). At the same time, VU-11 and VU-57B were cleaved with a lower rate and at the end of the incubation, the band of intact protein is clearly visible on the gel (Fig. 3B). In the absence of Ca^{2+}, CaM is cleaved by trypsin mainly at positions 90 and 106 [21]. These sites of cleavage are far from mutated Lys-75. Therefore, to explain the low rate of trypsinolysis of VU-11 and VU-57B, we have to postulate that mutations K75P and K75E have a long distance effect and decrease the rate of trypsinolysis in the C-terminal globular domain of CaM.

The data of native electrophoresis cannot be used for unequivocal determination of the nature of peptides accumulated during trypsinolysis. Electrophoretic mobility depends on the size, charge and form of peptides. In addition, peptides are differently stained and short peptides are lost during fixation and staining. Therefore, the data of Fig. 3 provide information only about the susceptibility of different mutants to trypsinolysis and cannot be used for exact identification of the peptide pattern. Our attempts to use gradient SDS-gel electrophoresis [23] for determination of the site of cleavage were also unsuccessful because many CaM peptides possessed an anomalous electrophoretic mobility and are badly stained on the SDS-gel. In order to determine the sites of trypsinolysis, we analyzed the mixture of CaM peptides by means of MALDI-MS. Since this method provides determination of the molecular weight with a precision of less than 1%, we were able to assign MS peaks to certain tryptic peptides of CaM. This approach was already successfully used to follow trypsinolysis of CaM and its mutants [22,24].

As expected, in the presence of Ca^{2+}, both wild-type CaM and SynCaM were cleaved by trypsin at positions 74/75 and 78 and two large peptides restricted by residues 1–74/75 and 78–148 were accumulated in the incubation mixture (Fig. 4A). In good agreement with earlier published results [21], additional sites of cleavage were detected at positions 30 and 37. A similar pattern of trypsinolysis was characteristic for VU-11 (K75P) and VU-57B (K75E), although in these two cases, the probability of cleavage at position 75 was reduced and these mutants were cleaved mainly at Lys-77 (Fig. 4A). VU-35 containing the additional tripeptide KGK in the middle of the central helix was cleaved at Lys-81 and 83, i.e. at the sites which are absent in the structure of wild-type CaM or SynCaM. These data once again indicate that the KGK insert is highly flexible and accessible to the tryptic attack. The pattern of tryptic peptides of VU-57A (K75A) and VU-57D (K75V) is very unusual. In these two cases, we failed to detect a large C-terminal peptide restricted by residues 78–148 of CaM (Fig. 4A). These mutants were preferentially cleaved at positions 30, 37, 106, 115 and 126, whereas the probability of a tryptic

Table 1
Primary structure of the central helix of bovine brain wild-type calmodulin, SynCaM and its mutants

Protein	Residues		
	67	75	90(93)
Wild-type	E-F-L-T-M-M-A-R-	K-M-K-D-T-D- - - -S-E-E-E-I-R-E-A-F-	R-V-F
SynCaM	E-F-L-*N*-*L*-M-A-R-	K-M-K-D-T-D- - - -S-E-E-E-*L*-*K*-E-A-F-	R-V-F
VU-11 (K75P)	E-F-L-*N*-*L*-M-A-R-	**P**-M-K-D-T-D- - - -S-E-E-E-*L*-*K*-E-A-F-	R-V-F
VU-35 (K75P+KGK)	E-F-L-*N*-*L*-M-A-R-	**P**-M-K-D-T-D-**K-G-K**-S-E-E-E-*L*-*K*-E-A-F-	R-V-F
VU-57A (K75A)	E-F-L-*N*-*L*-M-A-R-	**A**-M-K-D-T-D- - - -S-E-E-E-*L*-*K*-E-A-F-	R-V-F
VU-57B (K75E)	E-F-L-*N*-*L*-M-A-R-	**E**-M-K-D-T-D- - - -S-E-E-E-*L*-*K*-E-A-F-	R-V-F
VU-57D (K75V)	E-F-L-*N*-*L*-M-A-R-	**V**-M-K-D-T-D- - - -S-E-E-E-*L*-*K*-E-A-F-	R-V-F

Differences in the primary structure of wild-type CaM and SynCaM are marked in italic, a mutation in position 75 and insert KGK are marked in bold

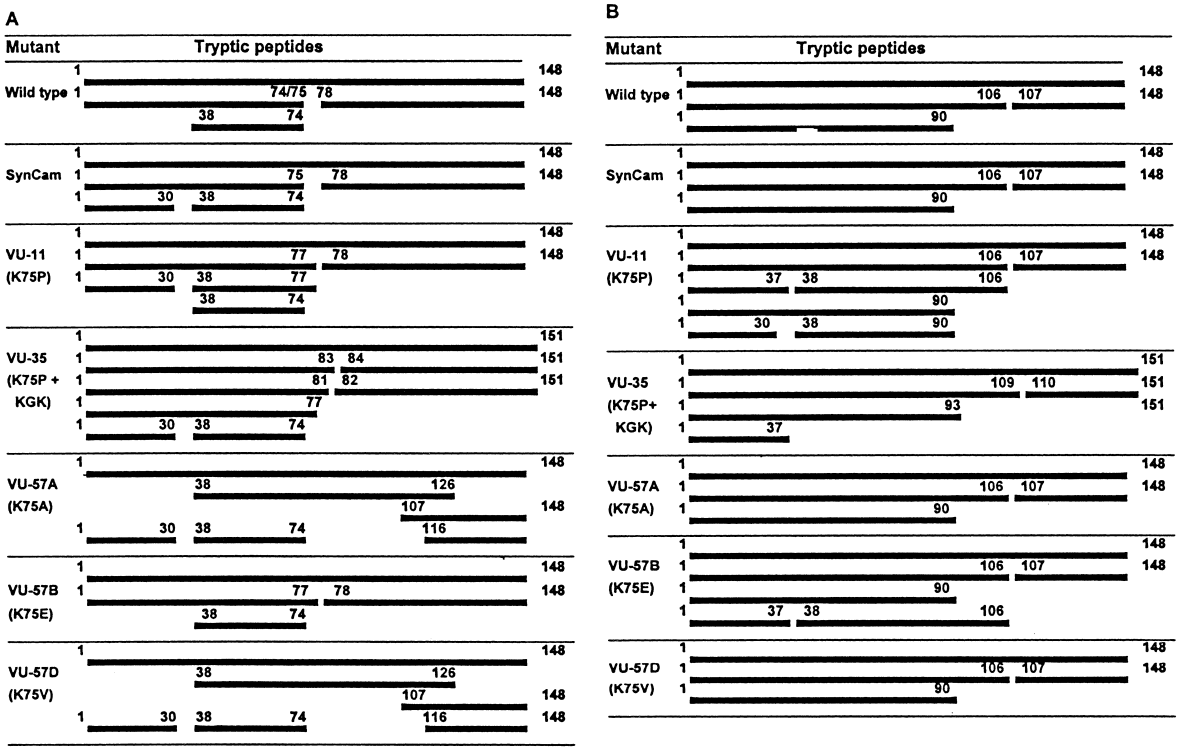

Fig. 4. Pattern of peptides obtained after trypsinolysis of calmodulin mutants in the presence of 1 mM $CaCl_2$ (A) or in the presence of 1 mM EGTA (B). Bars represent peptides with the numbers indicating amino acid residues.

attack at positions 74 and 77 was substantially reduced (Fig. 4A). As a result, the mixture of tryptic peptides of VU-57A and VU-57D contains a long central peptide (residues 38–126) and two short C-terminal peptides (residues 107–148 and 116–148) which are unusual for any other CaM mutants (Fig. 4A). These results indicate that replacement of Lys-74 by Ala or Val has a long distance effect and increases the susceptibility of the C-terminal sites of CaM (Arg-106 and Lys-115) to trypsinolysis.

In the absence of Ca^{2+}, both wild-type CaM and SynCaM were cleaved by trypsin at positions 90 and 106 (Fig. 4B). This agrees with earlier published data [21]. The overall pattern of cleavage of VU-57A (K75A) and VU-57D (K75V) is similar to that of SynCaM and the main sites of a tryptic attack are Arg-90 and 106 (Fig. 4B). VU-11 (K75P) and VU-57B (K75E) were also cleaved at position 90 and 106. In addition, two other sites (Arg-37 and Lys-30) were susceptible to proteolysis in these two mutants (Fig. 4B). It is worthwhile mentioning that in the absence of Ca^{2+}, the overall rate of trypsinolysis of VU-11 and VU-57B was significantly lower than for all other mutants of CaM (Fig. 3B). This means that replacement of Lys-75 by Pro or Glu somehow decreases the rate of proteolysis at positions 90 and 106. The pattern of tryptic peptides of VU-35 is similar to that of SynCaM. Indeed, VU-35 was cleaved at positions 93 and 109 which are homologous to Arg-90 and 106 of wild-type CaM and SynCaM. The rate of trypsinolysis of VU-35 was similar to that of SynCaM (Fig. 3B).

4. Discussion

Lys-75 is located in the very center of a flexible linker connecting two globular domains of CaM [2,25]. Residues Arg-74–Lys-77 form a turn of the central helix that is exposed to the solvent, especially in the Ca^{2+}-saturated CaM. Indeed, in the presence of Ca^{2+}, Lys-75 is highly reactive and easily modified by different fluorescent labels [26] and CaM is rapidly cleaved by trypsin at positions 74, 75 and 77 [21,22]. Therefore, one may suppose that replacement of Lys-75 will affect the flexibility of the linker and by this means affect the overall structure of CaM. The data presented confirm this suggestion.

All mutants analyzed in this paper can be divided into three groups. The first group is presented by VU-35. This mutant contains replacement K75P with simultaneous insertion of the KGK tripeptide between residues 80 and 81 of SynCaM (Table 1). Insertion of KGK makes the structure of the central helix of VU-35 more similar to that of troponin C, which also contains this tripeptide in a homologous position [25]. In the case of troponin C, the central helix is stabilized by the N-terminal helix, which is absent in the structure of CaM [25]. Therefore, we may expect that VU-35 will have a long unordered stretch of residues in the middle of the central helix (Fig. 1). In good agreement with this suggestion, we found that VU-35 has a more compact form during electrophoresis in the absence of Ca^{2+} (Fig. 2). In addition, this mutant is easily cleaved by trypsin both in the presence and in the absence of Ca^{2+} (Fig. 3) and the main sites of cleavage in the presence of Ca^{2+} are residues 77, 81 and 83 (Fig. 4).

VU-11 (K75P) and VU-57B (K75E) form the second group of mutants. These mutants are characterized by a partially-destabilized central helix. Mutation K75P decreases the probability of α-helix formation in the N-terminal part of the central helix (Fig. 1). A similar effect is expected for the K75E mutation since introduction of a negative charge in position 75 will cause electrostatic repulsion with the negative

charge of Asp-78 and by this means destabilize the C-terminal part of the central helix. Destabilization of the central helix may induce re-orientation of two globular domains with a concomitant change in their exposure to the solvent. This effect is demonstrated by a decreased susceptibility of VU-11 and VU-57B to trypsinolysis in the absence of Ca^{2+} (Fig. 3). Under these conditions, SynCaM and wild-type CaM are cleaved at positions 106 and 90 ([21] and Fig. 4). These residues are located in helices E and F, flanking the third Ca-binding site of CaM. Both these sites seem to be partially-protected by mutations K75P and K75E (Fig. 3B). At the same time, these mutations increase the probability of a tryptic attack at positions 30 and 37 of Ca-free CaM (Fig. 4B). Arg-37 is located in a flexible loop connecting the first and the second Ca-binding sites and is accessible to trypsinolysis [2,3,21]. Thus, mutations K75P and K75E partially stabilize the C-terminal and at the same time destabilize the N-terminal part of CaM. These results mean that the central helix is not a passive tether but plays an important role in transduction of a conformational signal between two globular domains of CaM.

The third group of mutants combines VU-57A (K75A) and VU-75D (K75V). Replacement of Lys-75 by Ala or Val makes the formation of hydrophobic contacts possible between Phe-68, Met-71 (or Leu-71), Met-72 and residues located in position 75. This will stabilize the N-terminal part of the central helix and this stabilization will be larger for VU-57D having a bulky and hydrophobic Val in position 75. In complete agreement with this suggestion, we found that Ca-saturated VU-57D is rather stable to trypsinolysis (Fig. 3A). The data of MALDI-MS (Fig. 4A) indicate that in the presence of Ca^{2+}, the central helix of both VU-57A and VU-57D is more resistant to trypsinolysis than the central helix of any other CaM mutants. Stabilization of the central helix of VU-57A and VU-57D is accompanied by destabilization of the C-terminal domain of these mutants. This is reflected by an increased probability of a tryptic attack at residues 106, 116 and 126 located in helices F and G and in the linker connecting the third and the fourth Ca-binding sites of CaM (Fig. 4A). These data also indicate the active role of the central helix in transduction of structural changes between two globular domains of CaM.

Summing up, we may conclude that the point mutations in the central helix have a long distance effect and evoke structural changes both in the N- and the C-terminal domains of CaM. Therefore, we may suppose that mutations at position 75 will affect the interaction of CaM with different target proteins. This problem is under investigation now.

Acknowledgements: The authors are grateful to Dr Alexander V. Vorotnikov (Institute of Experimental Cardiology, Russian Cardiological Research Center) for his help in the expression of calmodulin mutants and Dr Michail I. Titov (Institute of Bioorganic Chemistry, Russian Academy of Sciences) for his help in performing MALDI-MS. This investigation was supported by Grants from the Russian Foundation for Basic Research (Grant N 98-04-48116) and the Wellcome Trust.

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